REMARKS

Applicants respectfully request reconsideration of the present application in view of the foregoing amendments and in view of the reasons that follow.

Claims 1-20 are canceled.

Claim 41 and 42 are currently being added. Newly added claims 41 and 42 are added to further define claim scope.

Claims 21 and 31 are currently being amended.

This amendment adds, changes and/or deletes claims in this application. A detailed listing of all claims that are, or were, in the application, irrespective of whether the claim(s) remain under examination in the application, is presented, with an appropriate defined status identifier.

Exemplary support for the amendments to claims 21 and 31, is found in the specification on page 58, line 31, through page 60, line 26. Exemplary support for newly added claims 41 and 42 is provided throughout the specification. See, e.g., page 23, line 32, through page 24, line 7; page 23, lines 26-29 and page 58, line 31, through page 60, line 21.

After amending the claims as set forth above, claims 21-42 are now pending in this application.

Rejoinder

Applicants appreciate the examiner's summary of the law regarding rejoinder and reiterate their request that when an allowable product claim is identified, claims 32-34, 37-40 be added to the present application.

Rejection under 35 USC 112, second paragraph

Claims 21, 31, and 35-36 are rejected under 35 USC §112, second paragraph, as being indefinite for failing to point out and distinctly claim the subject matter which the applicants

regard as the invention. More specifically, with regards to claims 21 and 31 and dependant claims 35-36, the examiner believes that the phrase "naturally occurring" is indefinite. Applicants respectfully disagree with the examiner. However, to expedite prosecution applicants have amended claims 21 and 31 to remove the phrase "naturally occurring." Applicants believe that the pending independent claims 21 and 31, and dependant claims 35-36 are definite and cover the subject matter that applicants regard as the invention. Therefore, applicants argue that this rejection should be withdrawn and the present claims allowed.

Rejection under 35 USC §101

Claims 21-29, 31 and 35-36 are rejected under 35 USC §101 because the examiner alleges that the claimed invention is not supported by either a specific and substantial asserted utility or a well established utility. Applicants respectfully request reconsideration and withdrawal of the rejection.

MPEP §2107(II) states that "an applicant need only provide one credible assertion of specific and substantial utility for each claimed invention." Additionally, an applicant needs to "establish a probative relation between the submitted evidence and the originally disclosed properties of the claimed invention." On the basis of the above recited MPEP sections, the original specification and the attached publications, applicants argue that the subject matter of the present application possesses specific and substantial utility as required under 35 U.S.C. §101.

First, the original specification broadly identifies the utility of the invention on page 20, lines 30-33, reciting its use in diagnosing, treating or preventing transport/secretory, neurological, as well as other disorders. More specifically, the specification, starting on page 33, line 29 and ending on page 36, line 10, identifies the claimed polypeptides' use in diagnosing, treating, or preventing skin disorders, specifically Keratosis, and developmental disorders of the central nervous system (CNS). Furthermore, applicants attach herein three publications that desclose a membrane channel protein gene, GJB4, which has a 97% sequence similarity with claimed SEQ ID NO: 7.

The first article, Marcari et al., "Mutation in the Gene for Connexin 30.3 in a Family with Erythrokeratodermia Variabilis," *Am. J. Hum. Genet.*, 67:1296-1300 (2000), (Exhibit A), identifies GJB4 as a connexin polypeptide, which plays a role in the formation of gap junctions or arrays of aqueous intercellular channels connecting neighboring cells. This publication suggests that Erythrokeratodermia Verabilis (also called Keratosis Rubra Figurata), which is a disease characterized by the presence of hyperkeratosis, is genetically heterogenous and that mutation in GJB3, GJB4 and GJB5 likely indicated the presence of this disease. See Marcari et al., page 1297. A later article, Richard et al., "Genetic Heterogeneity in Erythrokeratodermia Variabilis: Novel Mutations in the Connexin Gene GJB4 (Cx30.3) and Genotype-Phenotype Correlations," *J. Invest. Dermatol.*, 120:601-608 (2003), (Exhibit B), continued studying GJB4 and confirmed the casual role of GJB4 mutations in Erythrokeratodermia Verabilis. See Richard et al., page 609. The findings of both of the above articles supports SEQ ID NO: 7's use in diagnosing and/or treating skin disorders, in particular, forms of Keratosis as described in the specification on page 33, line 32.

Additionally, Lopez-Bigas et al., "A Common Frameshift Mutation and Other Variants in GJB4 (Connexin 30.3): Analysis of Hearing Impairment Families," *Human Mutation*- Mutation in Brief #494 (2002) (Exhibit C), on page 4, suggests that along with its role in various forms of Keratosis, the GJB4 polypeptide is associated with hearing disorders like related connexin polypeptides, GJB1, GJB2, GJB3, and GJB6. As set forth above, the specification describes the detection of developmental disease of the central nervous system, which encompasses various hearing disorders, as another use for MECHP-7 polypeptides. Additionally, table 2 of the specification identifies SEQ ID NO:7 as a connexin.

In light of the above identified uses for the claimed protein sequence compositions, applicants argue that the subject matter of the claimed invention discloses at least "one credible assertion of specific and substantial utility" and thus, satisfies the requirements of 35 U.S.C. §101. Therefore, applicants argue that this rejection should be withdrawn and the present claims allowed.

Rejection under 35 USC §112, first paragraph

A. Enablement

The examiner, on page 11, first full paragraph, of the June 29, 2004 Office Action, argues that the claimed invention is not supported by either a specific and substantial utility or a well-established utility. As set forth above, applicants assert that the present application provides a specific and substantial as well as a well-established utility for the claimed invention. Applicants respectfully request reconsideration and withdrawal of the rejection.

Additionally, on page 11 second paragraph, the examiner again raises issue with the phrase "naturally occurring." Applicants, as explained previously, have addressed this concern and amended the claims to remove the offending phrase. Further, the examiner alleges that the present specification fails to disclose the specific biological activity that is associated with the claimed MECHP-7. Applicants respectfully, disagree with the examiner. The specification on page 58, line 31 through page 60, line 26, describes MECHP-7 as a membrane channel protein that maintains aquaporin function, protein transport function, gap junction activity, and ion channel activity. Additionally, Table 2 of the specification identifies MECHP-7 as a polypeptide that belongs to the connexin family as well as identifies the number of amino acid residues, the potential phosphorylation and glycosolation sites for SEQ ID NO:7, and the specific sequences contained within SEQ ID NO.:7. However, in order to expedite the prosecution of this application, applicants have amended claims 21 and 31 to include the phrase "and possessing membrane channel protein function."

In light of the above structural and functional description, one skilled in the art would be able to identify this polypeptide from the information provided, particularly with the help of the functional assay taught on page 60 line 29 through page 61, line 24 of the specification. Therefore, because utility has been established and the subject matter of the claims clarified and enabled, the 35 USC § 112, paragraph 1 rejection should be removed and the claims allowed.

B. Written Description

Claims 20-29, 31, 35-36 are rejected under 35 USC §112, first paragraph for not for not satisfying the written description requirement because the examiner alleges that the claimed variants are not properly described in the specification in such a way as to show the inventors possession of the invention at the time of filing. Applicants respectfully disagree with the examiner's conclusion and request reconsideration and withdrawal of the rejection.

As set forth in *Fiers v. Revel*, 984 F.2d 1164, 1171 (Fed. Cir. 1993), an adequate written description of DNA "requires a precise definition, such as by structure, formula, chemical name, or physical properties." The specification identifies the structure of the claimed sequences, teaching the entire amino acid and nucleotide sequences for SEQ ID NO:7 and SEQ ID NO:25, respectively, in the disclosed sequence listing. Additionally, Table 2 identifies the polypeptide as a Connexin, teaches the number of amino acid residues in the polypeptide sequence, lists the potential phosphorylation and glycosolation sites for SEQ ID NO:7, as well as describes the specific signature sequences contained within SEQ ID NO.:7. Further, on page 23, lines 12 – 13, the specific fragment useful for identifying the SEQ ID NO:25 is identified as the fragment from about nucleotide 656 to about nucleotide 700.

In addition to providing the above structural information, the specification and the amended claims describe the function of the claimed sequences as requested by the examiner. Particularly, the specification teaches that the claimed MECHP nucleotide sequence makes a functional MECHP polypeptide. Additionally, the claimed MECHP polypeptide, as set forth on page 58, line 31 through page 60, line 26 of the specification, is a membrane channel protein, specifically a connexin protein, possessing protein transport function, gap junction activity, and ion channel activity.

The disclosed structural and functional information described above supports that applicants were in possession of the claimed MECHP-7 polypeptide as well as its functional variants. Using techniques well known to those of skill in the art, as well as the functional assay disclosed on pages 58-60 of the specification, a person of ordinary skill in the art could

identify the claimed polypeptides. Finally, as explained above, applicants have amended claims 21 and 31 to include a functional description of the claimed varient, i.e. "possessing molecular channel protein function." In light of the structural and functional information contained in the present specification, applicants argue that the written description rejection, as well as the lack of enablement rejection, should be withdrawn and the present claims allowed.

CONCLUSION

Applicant believes that the present application is now in condition for allowance. Favorable reconsideration of the application as amended is respectfully requested.

The Examiner is invited to contact the undersigned by telephone if it is felt that a telephone interview would advance the prosecution of the present application.

The Commissioner is hereby authorized to charge any additional fees which may be required regarding this application under 37 C.F.R. §§1.16-1.17, or credit any overpayment, to Deposit Account No. 19-0741. Should no proper payment be enclosed herewith, as by a check being in the wrong amount, unsigned, post-dated, otherwise improper or informal or even entirely missing, the Commissioner is authorized to charge the unpaid amount to Deposit Account No. 19-0741. If any extensions of time are needed for timely acceptance of papers submitted herewith, Applicant hereby petitions for such extension under 37 C.F.R. §1.136 and authorizes payment of any such extensions fees to Deposit Account No. 19-0741.

Respectfully submitted,

Date 9/29/04

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Report

Mutation in the Gene for Connexin 30.3 in a Family with Erythrokeratodermia Variabilis

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Erythrokeratodermia variabilis (EKV) is an autosomal dominant keratinization disorder characterized by migratory erythematous lesions and fixed keratotic plaques. All families with EKV show mapping to chromosome 1p34-p35, and mutations in the gene for connexin 31 (Cx31) have been reported in some but not all families. We studied eight affected and three healthy subjects in an Israeli family, of Kurdish origin, with EKV. After having mapped the disorder to chromosome 1p34-p35, we found no mutations in the genes for Cx31, Cx31.1, and Cx37. Further investigation revealed a heterozygous T→C transition leading to the missense mutation (F137L) in the human gene for Cx30.3 that colocalizes on chromosome 1p34-p35. This nucleotide change cosegregated with the disease and was not found in 200 alleles from normal individuals. This mutation concerns a highly conserved phenylalanine, in the third transmembrane region of the Cx30.3 molecule, known to be implicated in the wall formation of the gap-junction pore. Our results show that mutations in the gene for Cx30.3 can be causally involved in EKV and point to genetic heterogeneity of this disorder. Furthermore, we suggest that our family presents a new type of EKV because of the hitherto unreported association with erythema gyratum repens.

Connexins (denoted by the prefix "Cx") are a family of polypeptides that form the subunits of the gap-junction channels. Members of the connexin family are characterized by four hydrophobic transmembrane domains (M1–M4) that are linked by one cytoplasmic and two extracellular (E1 and E2) loops. The N and C termini are located on the cytoplasmic membrane face. Extracellular-loop and transmembrane domains display the highest homology between the connexin family members, whereas the cytoplasmic loop and the C-terminal region are highly variable. Six connexin polypeptides assemble into a connexon, a hemichannel that interacts with its counterpart on adjacent cells to form a complete intercellular channel, thereby connecting the cytoplasm of neighboring cells (Yeager and Nicholson 1996). Gap junctions are composed of numerous aggregated connexons. Functionally, gap junctions allow rapid transfer, between adjacent cells, of ions and second messenger molecules of size <1 kD, thereby permitting a coordinated response of groups of cells to external stimuli. This cell-cell communication is crucial for growth control and differentiation, as well as for maintenance of tissue homeostasis (Goodenough et al. 1996). Signal transmission via gap junction is modulated by a number of molecular and physiological conditions. Channel permeability and voltage-gating properties depend on the composition of the connexons. So far, >14 connexin genes have been described, and each tissue expresses a specific subset of it. The importance of the physiological functions of connexins is illustrated by the identification of connexin mutations as the molecular cause of different human diseases (Krutovskikh and Yamasaki 2000). These results from human diseases are further corroborated by the findings obtained in knockout mice (White and Paul 1999).

Erythrokeratodermia variabilis (EKV [MIM 133200]) is a rare, autosomal dominant genodermatosis characterized by the coexistence of two morphological features: usually transient areas of erythema and keratotic lesions that are more or less fixed. The erythematous patches

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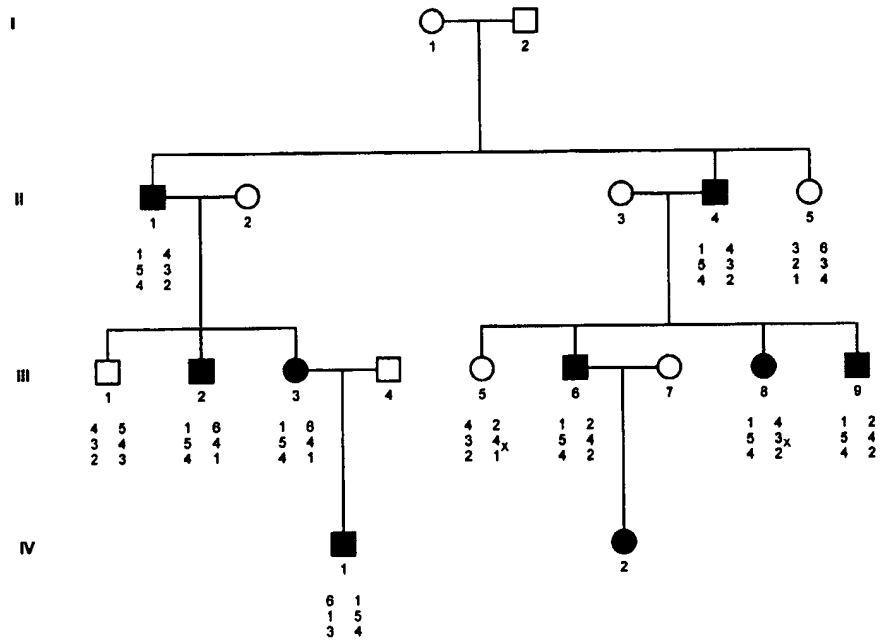

Figure 1 Pedigree and haplotype analysis of the family with EKV. Haplotypes are given (*top to bottom*) for markers D1S496, D1S472, and D1S186. Haplotype 1-5-4 was found to segregate with the disease. x = meiotic recombination.

vary in size, shape, and location and seem to be provoked by exposure to wind, cold, heat, and emotional stress (Rappaport et al. 1986). There are also fixed brownish-red hyperkeratotic plaques—particularly over the knees, elbows, groin, and axillae—in a symmetric distribution. There is a high intra- and interfamilial variability of the phenotype. Generally, EKV presents at birth or begins during early life. All families with EKV so far have shown mapping to chromosome 1p34-35, between the markers D1S496 and D1S186, with a maximum LOD score (Z_{max}) of 12.88 for D1S472 (Richard et al. 1997). These markers define a 2.6-cM candidate interval that contains a cluster of three genes for Cx: *GJB3*, encoding Cx31, *GJA4*, encoding Cx37, and *GJB5*, encoding Cx31.1 (van der Schroeff et al. 1988; Richard et al. 1997). Subsequently, six distinct Cx31 mutations were found—but only in 8 of 20 EKV families investigated, suggesting that EKV is genetically heterogeneous (Richard et al. 1998, 2000; Wilgoss et al. 1999). The human candidate region is orthologous to mouse chromosome 4, which harbors, in addition to the three genes described above, the gene for Cx30.3 (Hennemann et al. 1992). This suggests that this gene, *GJB4*, may be a good candidate. In this report, we describe an Israeli family, of Kurdish origin, with EKV, which we have also mapped to chromosome 1p34-35. Although we found no mutations in genes for Cx31, Cx31.1, and Cx37, we detected one, F137L, in the gene for Cx30.3. These findings provide the first and clear evidence for the causal involvement of a second connexin gene in this disorder and may delineate a specific subset.

An Israeli family of Kurdish origin that had already been described in 1978 (Hacham-Zadeh and Even-Paz 1978) was the object of our study. The pedigree included 32 individuals in four generations, of whom 8 affected and 3 healthy subjects were available for study (fig. 1). The dermatosis started at birth or shortly thereafter, and the patients were usually in good general health. Migratory erythematous lesions tended to gradually turn into more or less fixed keratotic plaques. Some of these erythematous lesions appeared as erythema gyratum repens, characterized by rapidly migrating figurate erythema 1–2 cm wide in an annular, garland, or spiral arrangement (Braun-Falco et al. 1984), which so far has not been reported in EKV (fig.2*B* and C). Generally, the palms and soles were intact. Lesions tended to become worse in summer and to improve in winter. In one of the patients, lesions also worsened during her pregnancies (fig. 2). Audiograms in two affected patients (III-6 and IV-1) were normal.

To see whether EKV in this family also shows linkage to 1p34-p35, as described elsewhere (Richard et al. 1997), linkage analysis with the three Généthon microsatellite markers D1S186, D1S496, and D1S472 was performed (Dib et al. 1996). The Z_{max} (2.343, at recombination fraction 0) of the two-point analyses, when

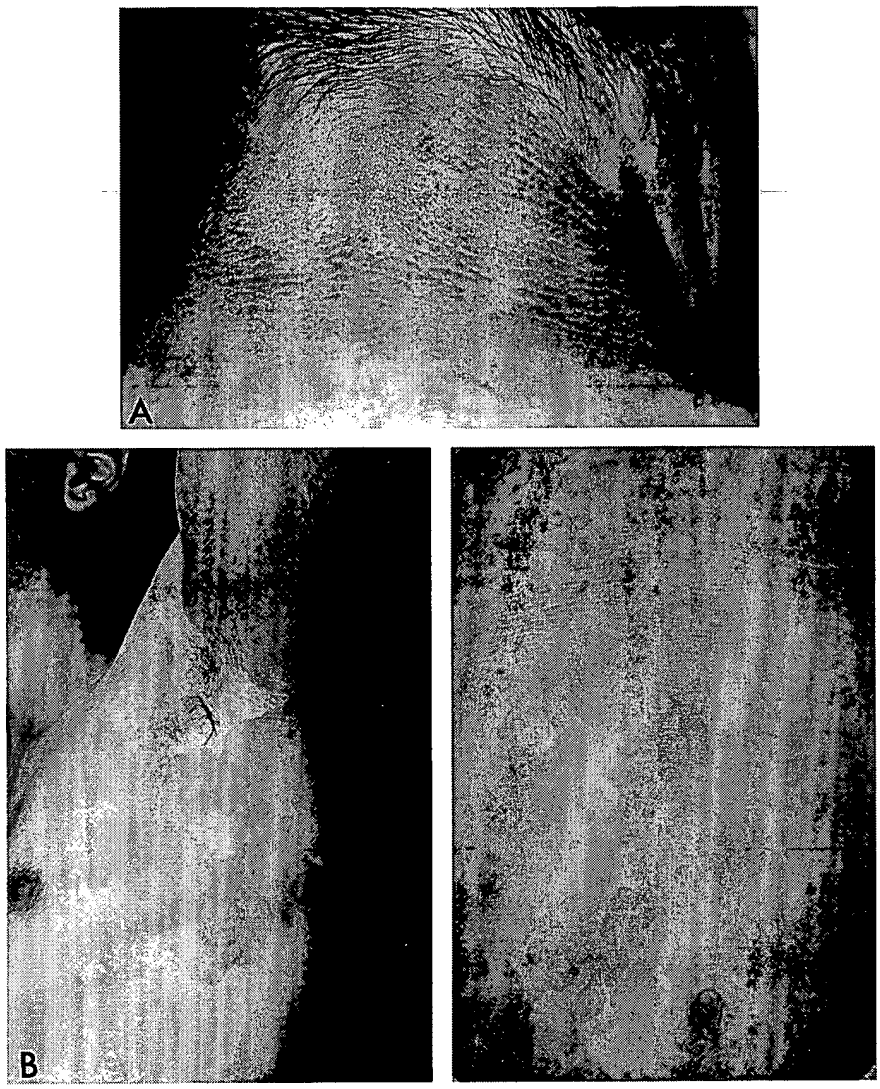

Figure 2 Clinical pictures. *A*, III-6, a 37-year-old man. The nape of the neck has brownish, lichenification-like hyperkeratotic plaque with mostly clear-cut borders. *B*, IV-1, a 19-year-old man. The periaxillary region has well-demarcated polycyclic plaque composed of diffuse scaling and erythema gyratum repens–like migratory lesions. *C*, IV-2, a 9.5-year-old girl. The anterior trunk has erythema gyratum repens–like migratory lesions.

MLINK from the LINKAGE 5.2 package (Lathrop et al. 1984) is used, was obtained with marker D1S472, and all affected individuals shared a common allele (fig. 1). The coding region of the genes for Cx37 (*GJA4*) and Cx31 (*GJB3*) were amplified as described elsewhere (Richard et al. 1997, 1998). The gene for Cx31.1 (*GJB5*) was amplified using primers Cx31.1-F (*5*′-AGAGCAAGTCTGTGATAAATGTAGG-3′) and Cx31.1-R (*5*′-CCCCTACCTCATGGCTTAGC-3′). Sequence analysis of the genes for Cx31 and Cx31.1 did not reveal any pathogenic mutation in two patients (II-1 and III-8) and one unaffected individual (II-5) in the family with EKV. In one patient (III-2), we identified, at position 388 of the gene for Cx37, a G→A transition that abolishes a *Bsi*EI restriction site. However, this previously reported polymorphism (Val130Ile) (Krutovskikh et al. 1996) did not cosegregate with the disease in the kindred with EKV, thus excluding the possibility that the disease is caused by this nucleotide change.

Mouse Cx30.3 transcripts are strongly expressed in keratinocytes, and the gene is localized within the connexin cluster on mouse chromosome 4 (Hennemann et al. 1992; Schwarz et al. 1992). This region is orthologous to human 1p34-p35, suggesting that the gene for

Cx30.3 might be a candidate in some cases of EKV. Searching the nonredundant database by BLASTN, at the UK Human Genome Mapping Project Resource Centre Web site, with the mouse gene for Cx30.3 (GenBank accession number M91443), we identified the human PAC clone RP1-34M23 (GenBank accession number AL121988), which contains the four genes for Cx—Cx31, Cx31.1, Cx37, and Cx30.3. Expression analysis by reverse transcription (RT)–PCR showed that the gene for Cx30.3 is indeed expressed both in human skin in vivo and in cultured epidermal keratinocytes but not in cultured skin fibroblasts and leukocytes (fig. 3). Therefore, a 952-bp fragment containing the entire coding region of the gene for Cx30.3 was amplified using primers Cx30.3-F (5′-CAATCGCACCAGCATTAAGGG-3′; nt −126 to −106) and Cx30.3-R (5′-TGATCTTATCTGCTGATCTCGCAG-3′; nt 803–826) in one affected individual (III-2) in the family with EKV. Sequence analysis detected a heterozygous T→C nucleotide transversion at position 409, resulting in a phenylalanine-to-leucine change at position 137 (F137L) (fig. 4*A* and *B*). This heterozygous missense mutation, which creates a new *SmlI* restriction site, was found only in affected individuals in this family and was not detected in either unaffected family members (fig. 4*C*) or 200 alleles from normal white individuals. Sequence analysis of the gene for Cx30.3 did not reveal any other nucleotide variations in 10 control alleles.

This mutation occurs within the third transmembrane domain, M3, of Cx30.3 and alters a phenylalanine residue that is highly conserved within the Cx family of proteins (fig. 5). The same mutation was previously reported in the homologous residue of the gene for Cx32 (residue F141L) in X-linked Charcot-Marie-Tooth disease (Rouger et al. 1997) and of the gene for Cx31 (residue F137L) in a sporadic case of severe EKV (Richard et al. 2000), providing evidence for the crucial function of this amino acid residue. Indeed, the M3 domain is known to be involved in the formation of the wall of the gap-junction pore and is at least partly implicated in the voltage gating of the channels. Closure of the channel occurs by the tilt of the M3 domain, which blocks the channel by moving small polar groups out of the lumen and permitting bulky phenylalanines into it (Bennett et al. 1991). It has been proposed that the ki-

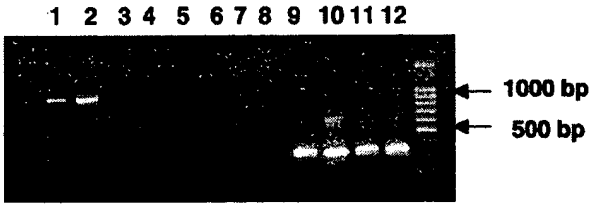

Figure 3 Expression analysis of the Cx30.3 transcript. *Lanes 1–4*, RT-PCR with Cx30.3 primers. *Lanes 5–8*, PCR with Cx30.3 primers, omitting RT. *Lanes 9–12*, RT-PCR with actin primers. Total RNA for the reactions was derived from human skin biopsy (*lanes 1, 5,* and *9*), cultured keratinocytes (*lanes 2, 6,* and *10*), cultured skin fibroblasts (*lanes 3, 7,* and *11*), and peripheral leukocytes (*lanes 4, 8,* and *12*).

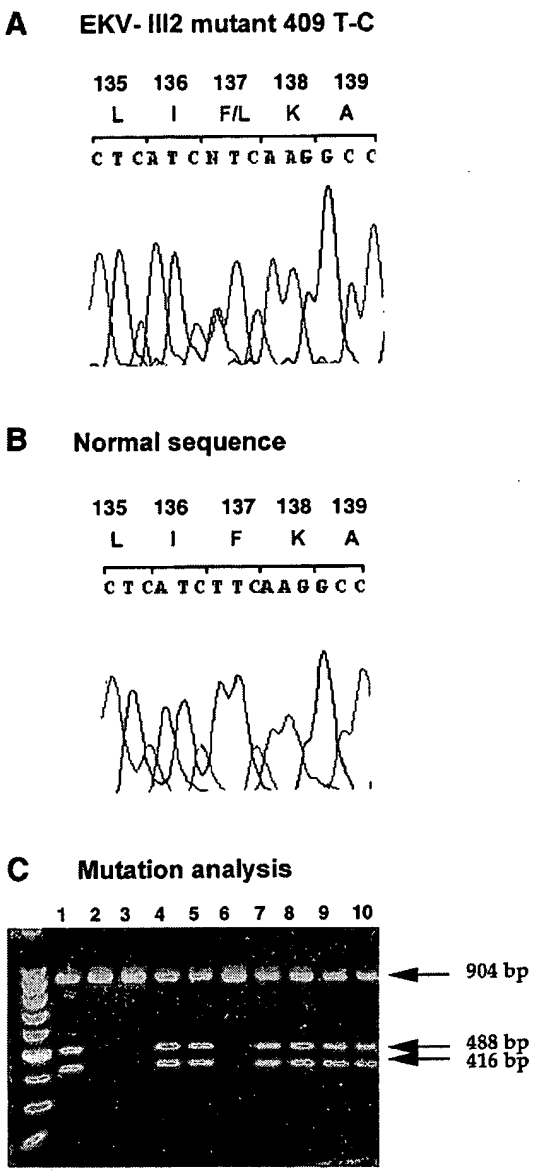

Figure 4 Molecular analysis of the gene for Cx30.3 (*GJB4*) in the family with EKV. *A* and *B*, Chromatograms of an affected individual with the Cx30.3 mutation, T409C, and a normal individual. *C*, Inheritance of the T409C mutation by restriction-enzyme digestion with *SmlI*. Normal alleles produce a 904-bp band that is cleaved into 488- and 416-bp fragments in mutant alleles. *Lanes 1–10*, Individuals II-1, II-5, III-1, III-2, III-3, III-5, III-6, III-8, III-9, and IV-1, respectively (see pedigree in fig. 1).

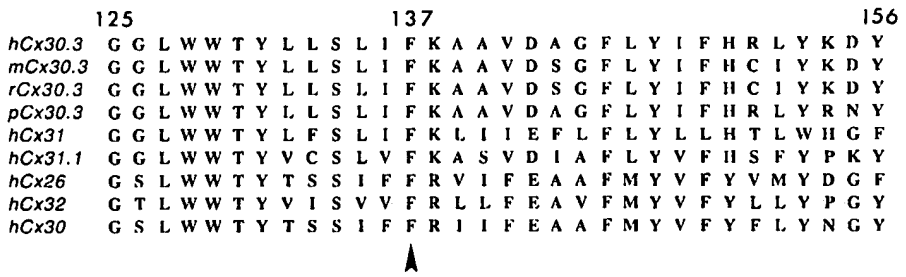

Figure 5 Sequence alignment of the M3 and partial E2 domains of human Cx30.3 with other β-class gap-junction proteins. The arrowhead indicates the position of the Cx30.3 mutation, F137L, in the family with EKV. GenBank accession numbers are CAB90270 (for hCx30.3), AAA37428 (for mCx30.3), CAA53762 (for rCx30.3), CAA06165 (for hCx31), AAD18005 (for hCx31.1), NP_003995 (for hCx26), B29005 (for hCx32), and NP_006774 (for hCx30). The sequence of pCx30.3 was obtained from Itahana et al. (1996). *h* = human, *m* = mouse, *r* = rat, and *p* = porcine.

netics of channel closure in response to voltage changes is inversely correlated with the number of phenylalanines in the M3 domain. Cx32 and Cx26, which harbor three phenylalanines, display slower kinetics than do Cx37 and Cx40, which have only one phenylalanine each. Cx30.3, with two phenylalanines, should have, according to this model, an intermediate channel-closure time (Unwin 1989; Hennemann et al. 1992). Hence, replacement of phenylalanine 137 by leucine may lead to faster closure of the channel, inhibiting the propagation of signals important for normal epidermal growth and differentiation. Two other mutations in the vicinity of Phe137, I141V and a deletion of Ile141, were identified in the M3 domain of Cx31 in recessive nonsyndromic hearing loss (Liu et al. 2000; Richard et al. 2000). Thus, mutations within the M3 domain may produce different phenotypes, such as hearing or skin disorders. This is not surprising, since Cx30.3 mRNA expression is restricted to skin and kidney in rodents, whereas the gene for Cx31 (*GJB3*) is also expressed in the inner ear and in the CNS (Hoh et al. 1991; Hennemann et al. 1992; Tucker and Barajas 1994).

The similarity in clinical pictures of patients with EKV who have mutations in the genes for Cx31 and Cx30.3 suggests that these two connexins are functional partners in the formation of keratinocyte gap junctions. Nevertheless, the cutaneous phenotype of EKV with erythema gyratum repens without palmoplantar involvement, which was observed in some patients from the family that we studied, may define a specific clinical subset and indicate subtle molecular and biological differences. Our results show that the connexin protein Cx30.3 is important for both the regulation of epidermal differentiation and the maintenance of epidermal homeostasis. Further investigations will be necessary to elucidate the specific role that each of the epidermally expressed connexins plays in the physiology and pathophysiology of epidermal tissues and in the clinical phenotype of connexin mutants.

Acknowledgments

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

GenBank Overview, http://www.ncbi.nlm.nih.gov/Genbank/GenbankOverview.html (for mouse gene for Cx30.3 and human clone RP1-34M23 [accession numbera M91443 and AL121988])
Généthon, http://www.genethon.fr (for genetic markers)
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/OMIM (for EKV [MIM 133200]).
UK Human Genome Mapping Project Resource Center, http://www.hgmp.mrc.ac.uk (for the BLAST search)

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Genetic Heterogeneity in Erythrokeratodermia Variabilis: Novel Mutations in the Connexin Gene *GJB4* (Cx30.3) and Genotype–Phenotype Correlations

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Erythrokeratodermia variabilis is an autosomal dominant genodermatosis characterized by persistent plaque-like or generalized hyperkeratosis and transient red patches of variable size, shape, and location. The disorder maps to a cluster of connexin genes on chromosome 1p34–p35.1 and, in a subset of families, results from mutations in the gene *GJB3* encoding the gap junction protein connexin-31 (Cx31). A recent report suggested the involvement of another connexin gene (*GJB4*) in the etiology of erythrokeratodermia variabilis. In this study, we sequenced the coding region of *GJB4* in 13 unrelated erythrokeratodermia variabilis families without detectable mutations in *GJB3*. Mutation analysis revealed six distinct missense mutations in five families and a sporadic case of erythrokeratodermia variabilis, all of which were not found in controls. Mutation G12D, identified in an extended Dutch family, lies in the predicted amino-terminus and may interfere with the flexibility of this domain, connexin selectivity, or gating polarity of gap junction channels. Other mutations (R22H, T85P, F137L, F189Y) were located in the transmembrane domains of Cx30.3, and are predicted to hinder regulation of voltage gating or alter the kinetics of channel closure. Affected individuals of two unrelated families harbored point mutations leading to amino acid substitution F137L, which was also reported in *GJB3*, yet the extent and severity of hyperkeratosis was milder compared to the corresponding mutation in *GJB3*. Two mutations (T85P, F137L) were associated with the occurrence of rapidly changing erythematous patches with prominent, circinate, or gyrate borders in affected children but not in adults, supporting the notion that this feature is specific to Cx30.3 defects. Nevertheless, we observed highly variable intrafamilial phenotypes, suggesting the strong influence of modifying genetic and epigenetic factors. In addition to pathogenic mutations, we identified several missense mutations and a 4 bp deletion within the *GJB4* coding region, which might represent either inconsequential polymorphisms or recessive mutations. In conclusion, our results demonstrate genetic heterogeneity in erythrokeratodermia variabilis, and emphasize that intercellular communication mediated by both Cx31 and Cx30.3 is crucial for epidermal differentiation. *Key words: connexin/epidermal differentiation/erythrokeratodermia variabilis/gap junction communication/mutation. J Invest Dermatol 120:601–609, 2003*

In 1925, Dutch dermatologist Mendes da Costa described the striking clinical features of a genodermatosis, which he named "erythro- et keratodermia variabilis" (EKV; MIM 133200) (Mendes da Costa, 1925). As reflected by this name, EKV is characterized by the presence of hyperkeratosis and extraordinarily variable areas of transient erythema that may coexist or occur independently. Some patients develop relatively fixed, yellow- or red-brown, hyperkeratotic plaques with well-demarcated, geographic borders that are symmetrically distributed over the extensor surface of extremities, buttocks, and lateral trunk. In others, hyperkeratosis is persistently severe and widespread, with accentuated skin markings, fine attached scaling, and, rarely, ichthyosis hystrix-like appearance. Patchy or diffuse palmoplantar keratoderma (PPK) is common (>50%), and areas of superficial peeling or hypertrichosis are not unusual (Richard, 2001). The hallmark of EKV is the continual occurrence of transient, sharply outlined, figurate red patches of variable intensity that fade within a few hours or days. The erythema

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Abbreviations: Cx, connexin; EKV, erythrokeratodermia variabilis; NT, amino-terminus; PPK, palmoplantar keratoderma.

is often triggered by sudden temperature changes or emotional stress, whereas hyperkeratosis may be provoked by chronic mechanical irritation of the skin (Richard, 2001).

EKV is inherited as an autosomal dominant trait and has been mapped in several extended families to a single genetic locus on human chromosome 1p34–p35.1 (van der Schroeff *et al*, 1984; Richard *et al*, 1997; Macari *et al*, 2000). The 2.6 cM candidate gene region was found to harbor four connexin (Cx) genes encoding the gap junction proteins Cx30.3 (*GJB4*), Cx31 (*GJB3*), Cx31.1 (*GJB5*), and Cx37 (*GJA4*) (Richard *et al*, 1997; Macari *et al*, 2000), which cluster within less than 40 kb. In 1998, Richard *et al* identified the *GJB3* gene and detected pathogenic missense mutations in four families with EKV, linking for the first time a defect in the cutaneous gap junction system to disturbed epidermal differentiation and function (Richard *et al*, 1998a). To date, a total of six distinct dominant *GJB3* mutations have been identified in eight unrelated EKV families (Wilgoss *et al*, 1999; Richard *et al*, 2000). All nucleotide changes lead to heterozygous amino acid substitutions, four of which (G12D, G12R, R42P, and C86S) were shown to alter gap junction communication and/or induce cell death *in vitro* (Di *et al*, 2002; Diestel *et al*, 2002; Rouan *et al*, in press). In addition, evidence emerged for an autosomal recessive variant of EKV caused by the homozygous missense mutation L34P (Gottfried *et al*, 2002). Nevertheless, as more than half of all tested EKV families did not harbor *GJB3* mutations, it was proposed that EKV might be genetically heterogeneous (Richard *et al*, 2000). This hypothesis was supported by genetic studies in a large Israeli-Kurdish EKV family, which revealed a heterozygous missense mutation (F137L) in the gene *GJB4* encoding Cx30.3, thus implicating yet another connexin gene in the pathogenesis of EKV (Macari *et al*, 2000).

Both Cx31 and Cx30.3 appear to be preferentially expressed in the upper differentiating layers of rodent and human epidermis and build with at least six other connexin constituents a complex and redundant gap junction network (Macari *et al*, 2000; Di *et al*, 2001; Richard, 2001). Gap junctions, which can be found in all tissues of the human body, are arrays of aqueous intercellular channels connecting neighboring cells. They allow direct cell–cell communication by rapid transfer of physiologic signals, ions, and small nutrients, which is pivotal for the coordination and synchronization of cellular responses to internal and external stimuli (Bruzzone *et al*, 1996). Gap junction channels are formed by connexins, a multigene family of at least 20 distinct integral membrane proteins in human (Willecke *et al*, 2002). Connexin monomers oligomerize to hexameric hemichannels (connexons), which accumulate at the plasma membrane where they dock head-to-head with connexons of apposing cells to complete a channel. As most tissues express several connexins, channels can be composed of connexons of the same type (homotypic), different types (heterotypic), or from mixed connexons (heteromeric) depending on their compatibility code, each with different channel permeability and gating properties. Each subunit is predicted to form four membrane-spanning α-helices (M1–M4) that may participate in forming the channel walls. Two extracellular domains (E1, E2) extend about 2 nm into the extracellular space, where they connect with their counterparts. Together with the cytoplasmic amino-terminus (NT), these domains are highly conserved among connexins, whereas the cytoplasmic central loop (CL) and carboxy-terminus are more variable and are assumed to determine connexin-specific attributes (Bruzzone *et al*, 1996).

The importance of gap junction intercellular communication for skin function and in particular epidermal differentiation is reflected by the large number of hereditary skin disorders that are caused by connexin gene mutations, including EKV, PPK associated with hearing loss, hidrotic ectodermal dysplasia (Clouston syndrome) (for review see Richard, 2001), and KID syndrome (Richard *et al*, 2002; van Steensel *et al*, 2002). To further explore the causal role of Cx30.3 gene defects in the pathogenesis of EKV and to elucidate genotype–phenotype correlations, we studied 13 unrelated EKV families that did not harbor detectable mutations in the Cx31 gene *GJB3*.

MATERIALS AND METHODS

Patients and biologic material We studied 57 patients affected with EKV from 13 unrelated families with their informed consent (108 individuals in total). Our cohort comprised five multigeneration families demonstrating linkage to the 1p locus (EKV1–5) (Richard *et al*, 1997; S. Morley, personal communication), four nuclear families (EKV6–9), and four sporadic cases (EKV10–13). With the exception of an African-American child, other patients were of northern and central European origin. The clinical diagnosis of EKV was established in at least one affected individual in each family by medical history, dermatologic examination performed by at least two dermatologists, and histopathologic evaluation of a skin biopsy. DNA samples from all participating individuals were obtained either from venous blood samples using standard extraction procedures or from buccal mucosa cells (Richards *et al*, 1993).

DNA amplification and mutation analysis The genomic sequence of *GJB4* was obtained from the published sequence of human clone RP1–34M23 assigned to chromosome 1p34.3–36.11 (GenBank accession number AL121988.10), which contains a contiguous cluster of four connexin genes including *GJA4*, *GJB3*, *GJB4*, and *GJB5*. The genomic organization of *GJB4* is similar to other connexin genes, including a first untranslated exon and a second exon that contains the entire coding information, separated by a single intron. For mutation analysis, using polymerase chain reaction (PCR) we amplified exon 2 and flanking untranslated regions in a 965 bp fragment using primers (1+) 5′-TCAATCGCACCAGCATTAAG-3′ and (2–) 5′-GGGGGACCTGTT GATCTTATC-3′ derived from the genomic sequence. PCR amplification was performed from genomic DNA using Taq DNA polymerase (Qiagen, Valencia, CA) and standard PCR conditions (94°C for 2 min; 36 cycles of 94°C for 30 s, 60°C for 50 s, 72°C for 1 min; and finally 72°C for 7 min). Amplicons were gel purified (QIAquick gel extraction kit, Qiagen) and directly sequenced using the BigDye terminator sequencing system on an ABI Prism 377 sequencer (PE Applied Biosystems, Foster City, CA). Sequence variants were confirmed by bidirectional DNA sequencing. In addition, we used restriction fragment analysis or denaturing high performance liquid chromatography (dHPLC) to verify the segregation of each mutation in the family of the proband and to exclude mutations from at least 184 population-matched control chromosomes. For restriction enzyme digestion with *Fau*I, *Mse*I, and *Tsp*45I we generated 587 bp amplicons, whereas 281 bp fragments were amplified for digestion with *Ava*II (primers available upon request). Amplicons were purified with MicroSpin™ S-400 columns (Amersham Pharmacia Biotech, Piscataway, NJ), digested for 2 h according to the supplier's recommended conditions (New England Biolabs, Beverly, MA), and analyzed on 6% nondenaturing TBE gels (Novex, San Diego, CA). For dHPLC analysis, we PCR-amplified *GJB4* in two overlapping fragments with primers (1+) and (3–) 5′-TTGTAGAGGCGGTGGAAGAT-3′ and (5+) 5′-CCTGTACGA CAACCTGAGCA-3′ and (2–), mixed each sample with a control sample in a ratio of 2:1 (vol/vol), denatured at 94°C for 10 min, cooled to 65°C for 10 min to allow heteroduplex formation, and maintained samples on ice until loading. Ten microliters of each sample were separately injected and analyzed on a WAVE™ DNA fragment analysis system (Transgenomic, Omaha, NE), over 7.5 min at temperature gradients of 62°C and 64°C that were established with the WAVE Maker program 3.4 (Transgenomic).

Electronic database information Online Mendelian Inheritance of Man (OMIM), http://www3.ncbi.nlm.nih.gov/Omim/searchomim.html; The Connexin-Deafness Homepage, http://www.iro.es/deafness/; The Human Gene Mutation Database, http://archive.uwcm.ac.uk/uwcm/mg/hgmd0.html; GenBank, http://www.ncbi.nlm.nih.gov/GenBank/GenBankSearch.html (for accession numbers AL121988 [*GJB4*]; Q9NTQ9 [hCx30.3]).

RESULTS

***GJB4* mutations and their corresponding phenotypes** We studied the clinical characteristics of EKV in nine families (EKV1–9) and four sporadic cases (EKV10–13) (**Table I**) and searched for disease-causing mutations in the coding sequence of

Table I. Clinical features of patients with EKV analyzed for *GJB4* mutations[a]

ID	Linked to 1p35	Generalized hyperkeratosis	Localized hyperkeratosis	PPK	Variable, transient erythema	Other features	*GJB4* mutation
EKV1	+		+		+		G12D
EKV2	+		+	+	+	Circinate or targetoid erythema	T85P
EKV3	+	+			+	Hyperpigmentation, hypertrichosis	F189Y
EKV4	+		+	+	+	Peeling; hypertrichosis	F137L
EKV5	+		+		+		–
EKV6		+			+	Hypertrichosis	–
EKV7			+		+		–
EKV8		+		+	+	CIE-like[b] fine, white scaling	–
EKV9			+		+	Peeling	R22H
EKV10			+	+ + +	+	Hypopigmentation, hypertrichosis	–
EKV11			+		+	Circinate, gyrate erythema	F137L
EKV12			+		+	Peeling; hypertrichosis	–
EKV13			+		+		–

[a]Without mutations in the coding sequence of *GJB3* (Cx31).
[b]CIE, congenital ichthyosiform erythroderma.

Table II. *GJB4* mutations identified in six families with EKV

Family ID	Nucleotide change	Codon	Connexin domain	Confirmation
EKV01	35 G/A	G12D	NT	*Tsp*45I
EKV02	253 A/C	T85P	M2	*Ava*II
EKV09	64 G/A	R22H	M1	dHPLC
EKV04	409 T/C	F137L	M3	dHPLC
EKV11	411 C/A	F137L	M3	*Mse*I
EKV03	566 T/A	F189Y	M4	dHPLC

Table III. *GJB3* and *GJB4* mutations previously identified in EKV

Gene	Nucleotide change	Codon	Connexin domain	Confirmation	Reference
GJB4	411C/A	F137L	M3	*Tsp*45I	Macari *et al* (2000)
GJB3	34G/C	G12R	NT	*Bsr*B1	Richard *et al* (1998)
GJB3	35G/A	G12D	NT	*Bsr*B1	Richard *et al* (1998)
GJB3	34G/A	G12S	NT	BsrB1	Richard (2001)
GJB3	101T/C	L34P[a]	M1	*Ban*I	Gottfried *et al* (2002)
GJB3	125G/C	R42P	M1/E1	*Bst*UI	Richard *et al* (2000)
GJB3	125G/C	R42P	M1/E1	*Hin*PII	Wilgoss *et al* (1999)
GJB3	256T/A	C86S	M2	*Nla*III	Richard *et al* (1998)
GJB3	256T/A	C86S	M2	*Nla*III	Richard *et al* (1998)
GJB3	409T/C	F137L	M3	*Mbo*II	Richard *et al* (2000)

[a]Recessive mutation.

GJB4. Each family presented with characteristic skin findings of EKV. The hyperkeratosis was confined to localized plaques in 10 families, and individuals of the remaining three families had more widespread involvement. In three families, patchy or diffuse, glove-like PPK was observed. Mutation analysis revealed six distinct heterozygous missense mutations (**Tables II, III; Fig 5**), each of them completely cosegregating with the disease phenotype but not being detectable in a panel of at least 92 unrelated Caucasian control individuals.

Family EKV1 Members of this family were descendants of an extended Dutch pedigree, which could be traced to a common ancestor seven generations ago, and was originally used to map EKV near the Rh blood group locus on 1p (van der Schroeff *et al*, 1984). All five affected individuals from three different branches of this family carried a heterozygous G-to-A transition at nucleotide 35 (from ATG start site) in codon 12, changing glycine (GGC) to a negatively charged aspartic acid (GAC) (**Fig 1*A***). This substitution affects a residue in the cytoplasmic NT domain of Cx30.3 that is highly conserved among all members of the β-connexin family across species, and can be expected to seriously compromise conformation and functional properties of Cx30.3. The mutation introduces a new recognition sequence for *Tsp*45I and thus alters the pattern of PCR amplicons of *GJB4* upon digestion (**Fig 1*A***). Clinically, all individuals harboring mutation G12D showed typical features of EKV with extensive, symmetric, well-demarcated hyperkeratotic plaques on extremities and trunk and independently occurring, short-lasting erythematous patches (**Fig 1*B***) without striking phenotypic differences.

Family EKV2 This family was composed of 30 members, 16 of whom were affected and harbored an A-for-C substitution at nucleotide 253 (**Fig 2*A***). This mutation replaced the conserved threonine 85 (ACG) residue with proline (CCG) and created a new *Ava*II cut site used for restriction fragment analysis, which confirmed the cosegregation of T85P with the EKV phenotype (**Fig 2*A***). Structural predictions suggested that mutation T85P falls within the second transmembrane region M2, which is thought to contribute to the lining of the channel pore and regulation of voltage gating (Veenstra, 1996). The clinical presentation of EKV was highly variable within this family, but most individuals showed at examination relatively fixed hyperkeratotic plaques (see below).

Family EKV3 In this family, we detected a T-to-A substitution at nucleotide 566 leading to a nonconservative change of phenylalanine 189 (TTC) to tyrosine (TAC), which was confirmed by dHPLC analysis (**Fig 3*A***). Mutation F189Y eliminates another conserved phenylalanine residue in the fourth membrane domain of Cx30.3, which might potentially interfere with connexon assembly (Bruzzone et al, 1996). In contrast to other families, the prevailing feature in EKV3 was severe hyperkeratosis with accentuated skin markings, ridging in large skin folds, and noticeable hypertrichosis lanuginosa (**Fig 3*B***), but sparing palms, soles, and scalp.

Family EKV4 In this family, dHPLC and mutation analysis revealed a T-to-C transition of nucleotide 409 (**Fig 4*A***). The mutation, which has been previously observed in another EKV family (Macari *et al*, 2000), is predicted to replace a strictly conserved phenylalanine residue 137 (TTC) in the third transmembrane domain of Cx30.3 with leucine (CTC). This amino acid substitution could potentially alter topogenic assembly of connexons or kinetics of channel closure (Macari *et al*, 2000). In all affected individuals, we noted localized hyperkeratotic plaques with foci of superficial peeling and red patches mostly confined to the hyperkeratotic areas (**Fig 4*B–D***).

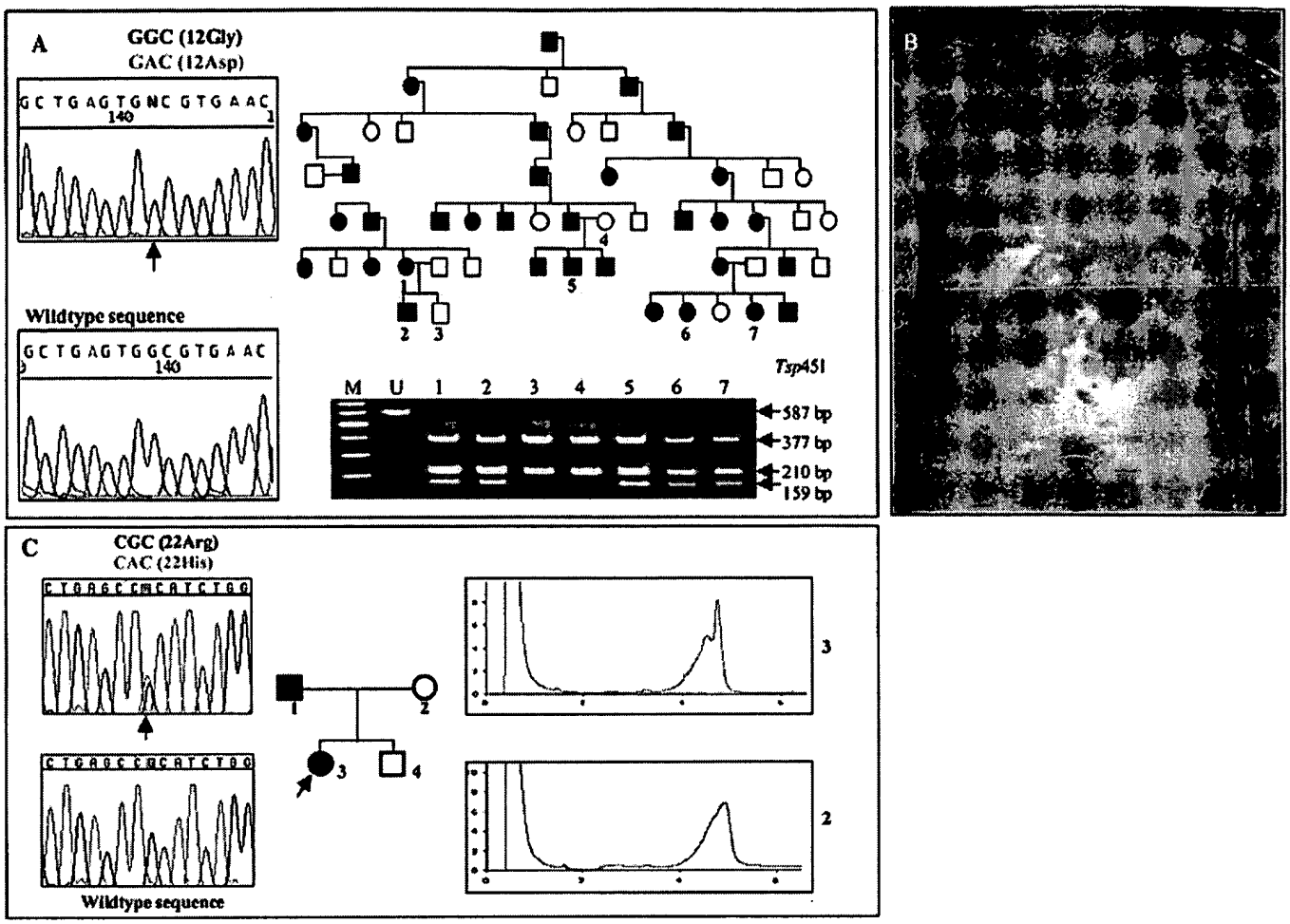

Figure 1. Mutations G12D in EKV1 and R22H in EKV9. (*A*) Sequence analysis reveals a G-to-A transition that substitutes glycine 12 with aspartic acid (*top left panel*) compared to the normal sequence (*bottom left panel*). Restriction fragment analysis with *Tsp*451 (*lower right panel*) confirms cosegregation of this mutation with the EKV in the family (*upper right panel*). The mutation introduces a new cut site, resulting in additional DNA bands of 159 bp and 51 bp (not shown) in affected individuals. M, 100 bp marker; U, undigested control; lane numbers correspond to individuals in the pedigree. (*B*) Variable erythematous patches within well-demarcated, symmetrically distributed hyperkeratotic plaques on the frontal trunk of individual EKV1-5. (*C*) Pedigree of EKV9 (*middle panel*) and sequence panels (*top left*) showing the G-to-A transition replacing arginine 22 with histidine and the normal sequence (*bottom left*). dHPLC analysis demonstrates an additional peak in the melting curve (EKV9-3) due to heteroduplex formation in the presence of the mutation (*top right*) compared to a control sample (*bottom right*).

Family EKV9 Both affected individuals EKV9-1 and EKV9-3 carried a heterozygous G-to-A substitution replacing arginine 22 (CGC) with histidine (CAC) in the first transmembrane domain of Cx30.3. This mutation R22H was not present in unaffected family members or in unrelated controls as determined by dHPLC analysis (**Fig 1C**). The clinical features included symmetric hyperkeratotic plaques on the extremities without PPK and faint underlying erythema as well as transient red patches. The affected parent EKV9-1 noticed a marked age-related regression of both morphologic components.

Family EKV11 The affected child EKV11-3 in this family harbored a C-to-A transversion of nucleotide 411, thus resulting in amino acid substitution F137L, similar to family EKV4. This mutation, which generates a new *Mse*I cut site, was not detected in DNA samples of either unaffected parent, suggesting that it has arisen *de novo* in this patient or reflects germline mosaicism (**Fig 4*E***). Initially, the proband had well-defined, hyperkeratotic plaques symmetrically distributed over the lower extremities and buttocks (**Fig 4*F***). The hyperkeratosis progressively worsened resulting in generalized involvement at the age of 5 y. From 3 mo of age, autonomous erythematous patches with prominent, raised, circinate borders and rapidly changing configuration were noted, leading to a garland-like appearance (**Fig 4*F, G***).

In the remaining seven EKV families, one of which links to the connexin cluster on 1p (S. Morley, personal communication), we were unable to detect pathogenic mutations in *GJB3* or *GJB4* and the molecular cause of EKV in these families still remains elusive. A retrospective critical evaluation of their clinical phenotype revealed that none of the patients had circinate or gyrate erythema as seen in several families segregating *GJB4* mutations. Considerable interfamilial phenotypic variability, as illustrated for families with *GJB4* mutations above, was also observed within this subgroup of seven families complicating direct clinical comparisons (see **Table I**). Nevertheless, we did not observe any discriminatory or consistently deviant clinical features of EKV that would allow us to clinically differentiate these patients from others harboring mutations in *GJB4* or *GJB3*.

Variable intrafamilial expressivity of *GJB4* mutations In family EKV2, clinical studies of 16 individuals with EKV from three generations carrying mutation T85P revealed marked intrafamilial variability of clinical features and striking age-dependent differences. Of the affected individuals, five adults between 23 and 70 y of age had no erythematous component at the time of examination (**Fig 2*C***) but all had a history of "blotching" and recurring red patches during childhood. In

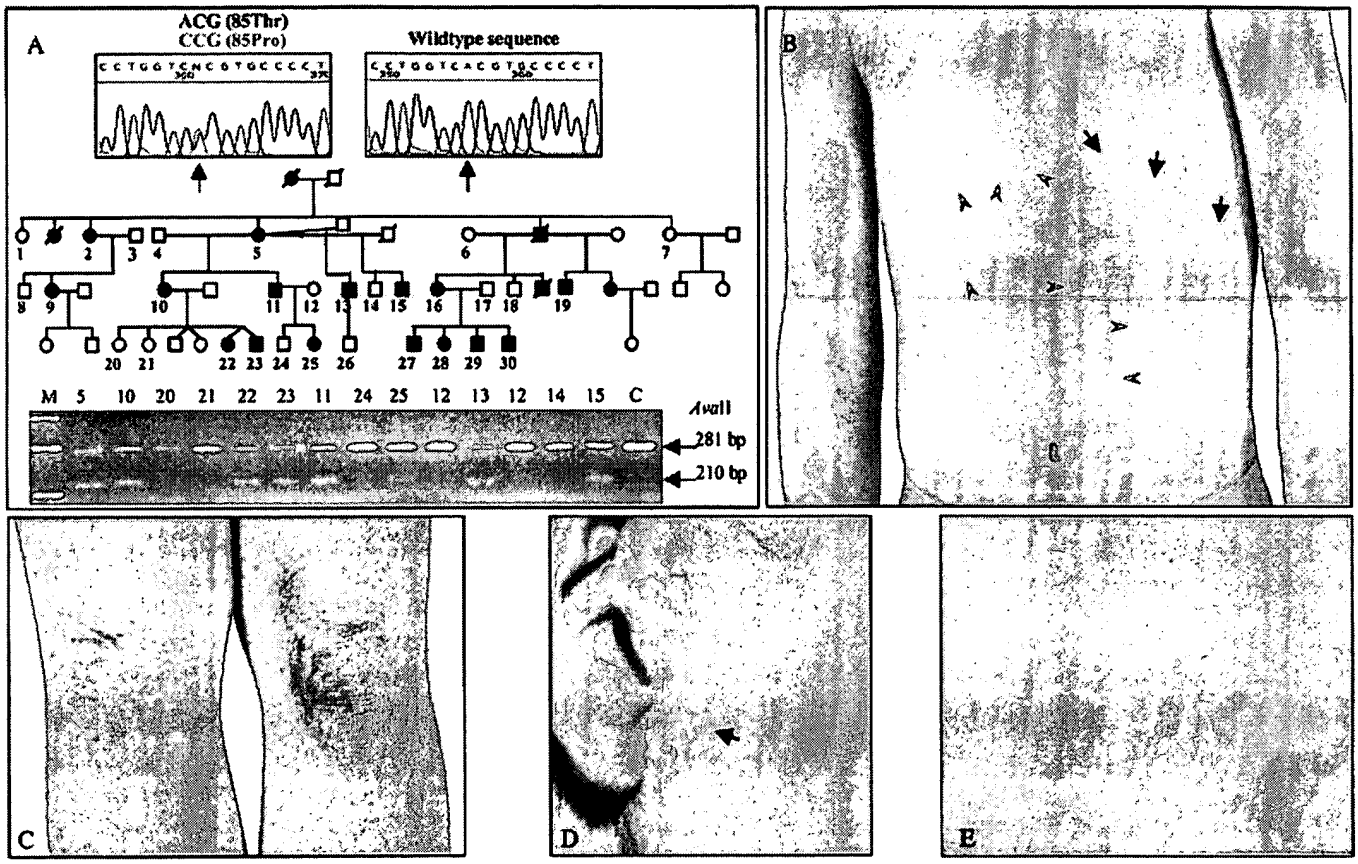

Figure 2. Mutation T85P in EKV2. (*A*) The sequence chromatogram of affected individual EKV2-5 depicts a heterozygous A-to-C transversion leading to missense mutation T85P in comparison to a control (*top panel*). This mutation creates a new recognition site for *Ava*II and results in two additional DNA bands of 210 bp and 71 bp (not shown) in affected individuals (*lower panel*). The lane ID numbers correspond to individual ID numbers in the pedigree of EKV2 (*middle panel*). M, 100 bp marker; C, control. (*B*) Extensive hyperkeratotic plaques on the frontal trunk and arms of an 8-y-old boy (EKV2-30). Note the target-like (→) and circinate (▷) erythematous patches indicated by *arrows*. (*C*) Fixed, hyperkeratotic plaques over both knees without erythema in individual EKV2-13. (*D*) Preauricular, sharply demarcated erythematous patch occurring during 15 min cold exposure of individual EKV2-2. (*E*) Close-up of erythematous patches with circinate borders in individual EKV2-30.

contrast, five out of 11 children and adolescents aged 2–16 y showed widely distributed, rapidly changing erythematous patches. In four of them, the erythema had well-defined, circinate borders and assumed annular, targetoid, or garland-like shapes (**Fig 2*B*, *E***). Extent and localization of hyperkeratosis differed from person to person and during the course of disease. In most affected individuals (10 of 16), hyperkeratosis was limited to a few plaques over large joints (**Fig 2*C***), whereas four others had larger plaques on extremities and trunk and mild PPK, covering > 25% of the body surface. Finally, one individual (EKV2-2) had neither hyperkeratosis nor erythema when examined at 60 and 62 y of age. She carried mutation T85P, had an affected child (EKV2-9), and was reported to have irregular red spots on exposed skin areas during the winter season. Indeed, well-demarcated, erythematous patches with irregular outline could be experimentally provoked when she spent 15 min in a cold room at 4°C (**Fig 2*D***), although limited local cold exposure (ice cube test) did not elicit an abnormal skin reaction. Collectively, these observations in family EKV2 illustrate intrafamilial clinical heterogeneity and reduced expressivity of the T85P mutation and underscore that the phenotypic expression of a dominant connexin mutation may critically depend on the individual's genetic background and other epigenetic (e.g., age, stress, climate) factors.

Identification of common sequence variants in *GJB4* In addition to the pathogenic mutations delineated above, we identified a series of sequence variants that did not cosegregate with EKV or were only found in the control cohort (**Table IV**). The silent sequence variant 369G/A (K123K) was found in one affected member of EKV3, whereas an unaffected member of the same family carried the heterozygous nucleotide substitution 611A/C resulting in nonconservative replacement of glutamic acid 204 with alanine (E204A). Glu204 is a charged residue at the boundary between the fourth transmembrane domain and the cytoplasmic carboxy-terminus tail domain, invariably present at this position in connexins of different species. Other missense mutations solely detected in unaffected controls in a heterozygous state included R124Q and C169W. Whereas the former amino acid substitution falls within the highly variable cytoplasmic loop, cysteine 169 is one of three highly conserved cysteines in the second extracellular loop that stabilize connexon structure by forming intramolecular disulfide bonds (Foote *et al*, 1998). Most interestingly, we identified in six out of 92 control individuals a heterozygous 4 bp deletion starting from nucleotide 154 on one allele of *GJB4* (**Fig 5*A***). This deletion, which could be easily detected by HPLC analysis under nondenaturing conditions (**Fig 5*B***), leads to frameshift and introduces a premature termination codon 54 residues downstream of the mutation site. The transcript containing the premature termination codon may be rapidly degraded through nonsense mediated RNA decay (Frischmeyer and Dietz, 1999), and the deletion may generate in essence a null allele. Despite these predictions, the 4 bp deletion was the most common sequence variant of Cx30.3 seen heterozygously in

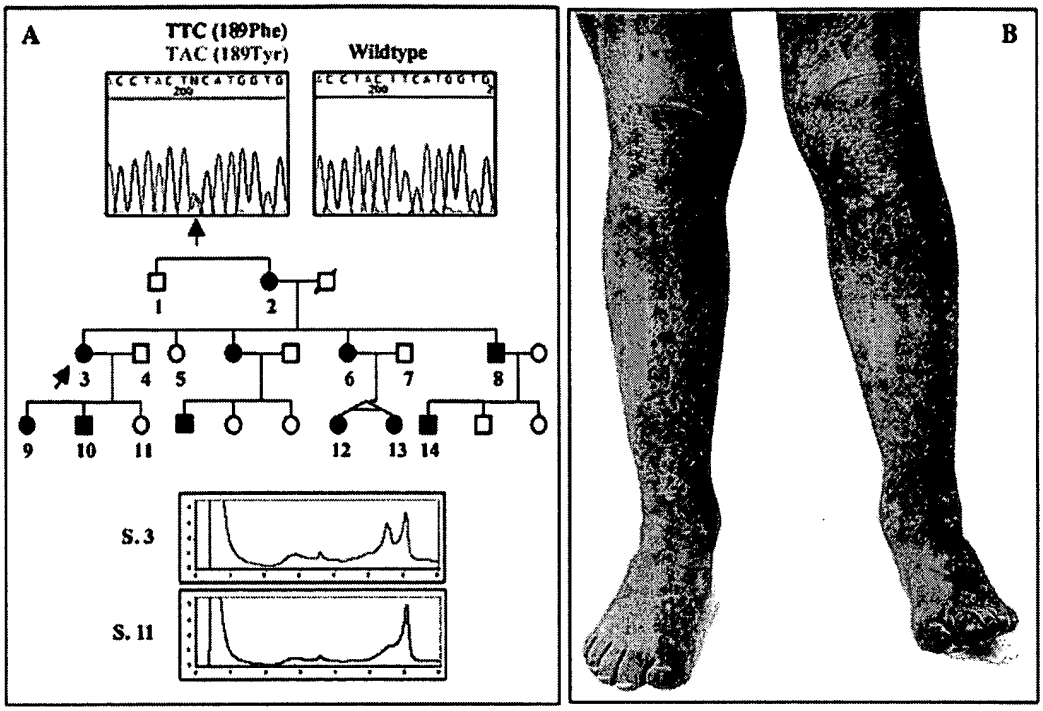

Figure 3. Mutation F189Y in EKV3A. Compared to the wild-type allele, sequence analysis demonstrates a heterozygous T-to-A transversion resulting in missense mutation F189Y (*top panel*). dHPLC analysis reveales heteroduplex formation (double peak) in a PCR amplicon of individual EKV3-3 (pedigree in *middle panel*), which is not present in amplicons of the unaffected individual EKV3-11 (single peak, *lower panel*). (*B*) Generalized hyperkeratosis with white, attached scale, hypertrichosis, and ridging over the knees with few areas of erythema in individual EKV3-13.

3.3% of all tested individuals, and not associated with a cutaneous phenotype.

DISCUSSION

We report here six distinct point mutations in the Cx30.3 gene *GJB4*, five of which have not been reported before, in five families and a sporadic case of EKV. Our results firmly establish the causal role of *GJB4* mutations in EKV and demonstrate that this rare disorder of cornification is genetically heterogeneous and can result from mutations in the two closely related epidermal connexin genes *GJB3* and *GJB4*. A comparison of the nature and location of mutations affecting Cx31 and Cx30.3 reveals striking similarities. All autosomal dominant point mutations lead to nonconservative amino acid substitutions, which apparently cluster (i) at Gly12 in the cytoplasmic NT domain, and (ii) in the membrane-spanning α-helices (**Fig 6**). Gly12 lies within a conserved window of six residues. It is shared between all β-type connexins, and it is one of four discriminatory residues that fundamentally differ in their chemical and physical properties between the α- and β-connexins (M. Falk, personal communication).

To date, pathogenic mutations involving residues 11 and 12 have been reported in no less than five different β-connexin genes, giving rise to Charcot–Marie–Tooth disease (G12S and S11G in Cx32), Clouston syndrome (G11S in Cx30), KID syndrome (G12D in Cx26), and EKV (G12D, G12R in Cx31, and G12D in Cx30.3) (Bergoffen *et al*, 1993; Richard *et al*, 1998a; 2002; Lamartine *et al*, 2000). This clustering of mutations points to a specific function for the nonpolar glycine at this position. Its replacement with a highly polar residue significantly changes the net charge of the NT tail, which is a crucial part of the transjunctional voltage sensor of gap junction channels, and may thus greatly alter ion permeation (Verselis *et al*, 1994; Purnick *et al*, 2000a; 2000b). In addition, Gly12 seems to determine conformation and flexibility of the NT. Nuclear magnetic resonance analysis of a synthetic peptide corresponding to the NT of Cx26 revealed the presence of a flexible hinge (open turn) initiated by Gly12, which is thought to permit the placement of the distal end of the NT into the channel pore (Purnick *et al*, 2000a). Substitution of Gly12 with residues predicted to decrease flexibility at this area (G12S, G12Y, G12V) completely compromised the function of Cx32 channels, whereas substitution with proline (G12P) had no significant effect (Purnick *et al*, 2000a). Moreover, there is evidence that a sequence motif in the NT is involved in determining the connexin selectivity and hetero-oligomerization (Falk *et al*, 1997). It is likely that Gly12 mutations of Cx30.3 and Cx31 in EKV interfere with one or more of these mechanisms and thus alter connexin function and/or gap junction communication in the epidermis.

Insight into mutation-specific differences of functional implications have also emerged from *in vitro* studies in HeLa cells expressing the Cx31 mutants G12D and G12R. Whereas both mutant variants of Cx31 formed normal-appearing gap junctional plaques at points of cell–cell contacts, dye transfer and calcium monitoring experiments demonstrated that G12D-Cx31 channels were nonfunctional (loss of function) (Rouan *et al*, in press). In contrast, G12R-Cx31 resulted in a significant increase of intercellular dye coupling (gain of function) possibly due to defective closure of gap junction channels and subsequent cell death (Diestel *et al*, 2002). Another study in NEB1 keratinocytes revealed evidence for impaired intracellular trafficking of Cx31 mutants (Di *et al*, 2002). Collectively, these observations suggest that any deviation from normal connexin and gap junction function can have detrimental consequences for intercellular communication and survival of epithelial cells.

All other mutations resulting in an EKV phenotype, several of which were observed in more than one family (R42P, C86S, F137L), are localized in the phylogenetically conserved transmembrane domains of Cx31 and Cx30.3 (**Fig 6**). These α-helical segments anchor the protein in the plasma membrane and contribute to the wall of the channel pore, regulating voltage gating and channel closure. Many dominant connexin mutations

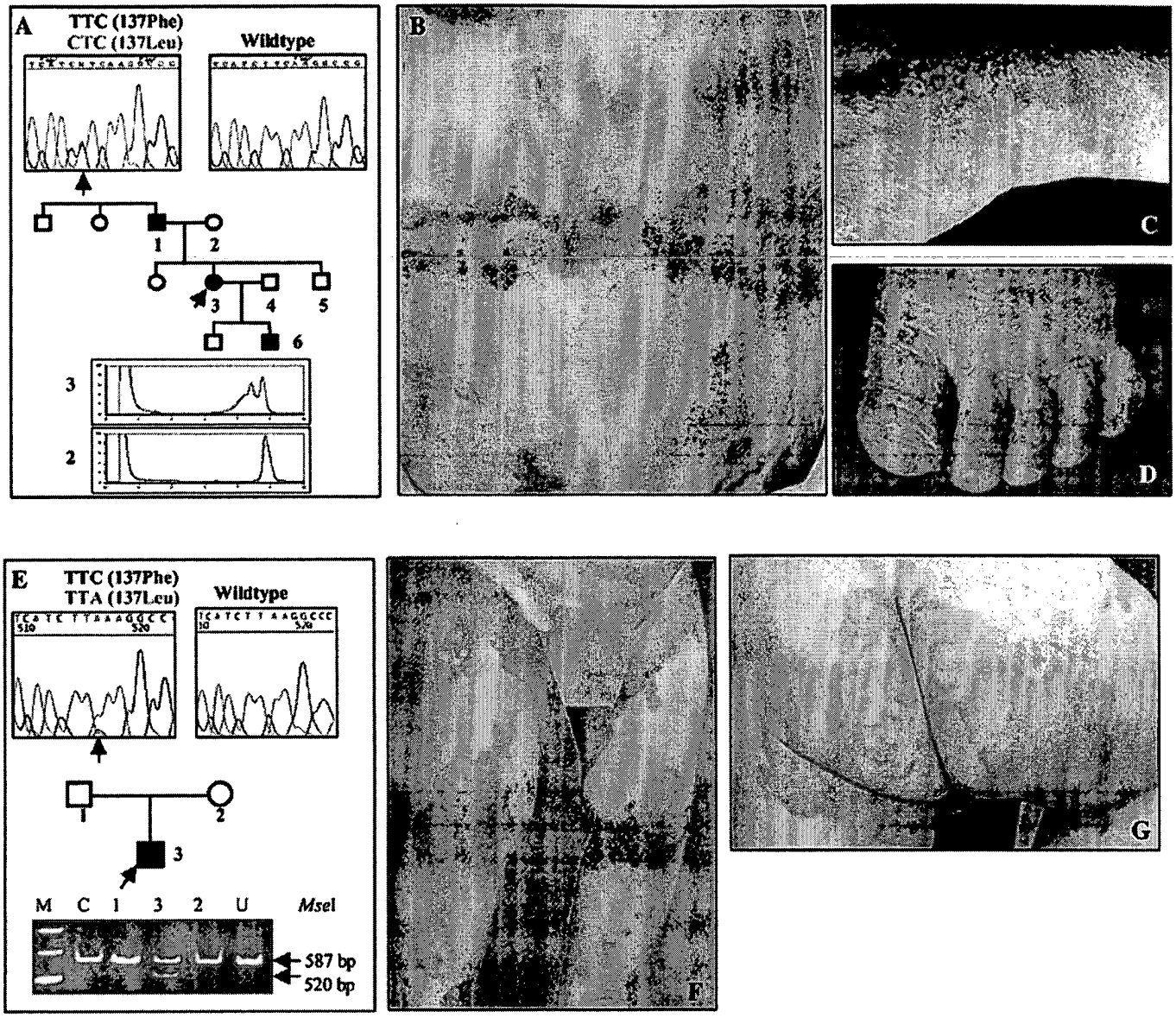

Figure 4. Mutations F137L in EKV4 and EKV11. (*A*), (*E*) Sequence chromatograms illustrate T-to-C substitution in EKV4 (*panel A*) and C-to-A substitution in EKV11 (*panel E*), each of which resides in codon 137 of *GJB4* and replaces phenylalanine with leucine (*top panels*). The presence of transition 409T/C is indicated by a second peak in the dHPLC profile of proband EKV4-3 (*A*, *lower panel*). The transversion 411T/A induces a new recognition sequence for *MseI*, thus creating upon digestion two new DNA bands of 520 bp and 67 bp (not shown) in the mutant allele (*E*, *lower panel*). M, 100 bp marker; C, control; U, undigested control; lane numbers correspond to individuals in the pedigree. (*B*) Figurate outlined, brownish hyperkeratotic plaques with erythematous areas on the abdomen of individual EKV11-6. (*C*), (*D*) Acral superficial peeling of the stratum corneum of individuals EKV4-3 and EKV4-6. (*F*), (*G*) Symmetrical, sharply demarcated, hyperkeratotic plaques on the legs and buttocks of a 2-y-old boy (EKV11-3). Note the circinate erythema with prominent borders.

Table IV. *GJB4* sequence variants of unknown biologic relevance and polymorphisms in unaffected controls

Nucleotide change	Codon	Connexin domain	Allelic frequency (%)	Reference
154delGTCT	PTT + 54	E1	3.3	This study
			4.5	Lopez-Bigas *et al* (2002)
307C/T	R103C	CL	0.8	Lopez-Bigas *et al* (2002)
369G/A	K123K	CL	0.01	This study
371G/A	R124Q	CL	0.005	This study
			0.8	Lopez-Bigas *et al* (2002)
478C/T	R160C	E2	0.4	Lopez-Bigas *et al* (2002)
507C/G	C169W	E2	0.01	This study
			0.4	Lopez-Bigas *et al* (2002)
611A/C	E204A	M4	N.D.	This study
			0.4	Lopez-Bigas *et al* (2002)

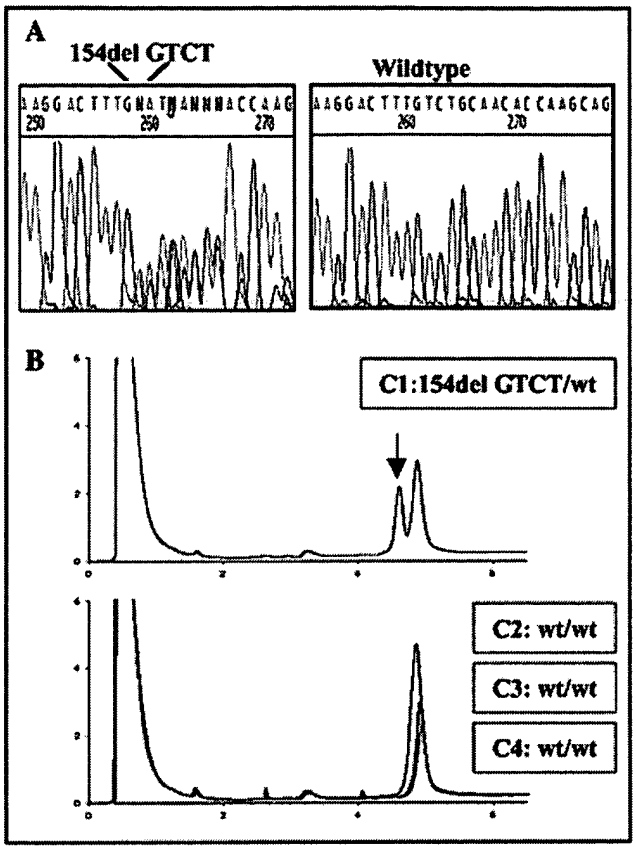

Figure 5. A common frameshift polymorphism of *GJB4*. (*A*) Sequencing analysis reveals a heterozygous deletion of 4 bp (154del4) resulting in frameshift in a control individual without a cutaneous phenotype (*left*) compared to the wild-type sequence (*right*). (*B*) The size difference between the mutant (*top*) and wild-type (*bottom*) allele is easily detectable by HPLC under nondenaturing conditions (50°C).

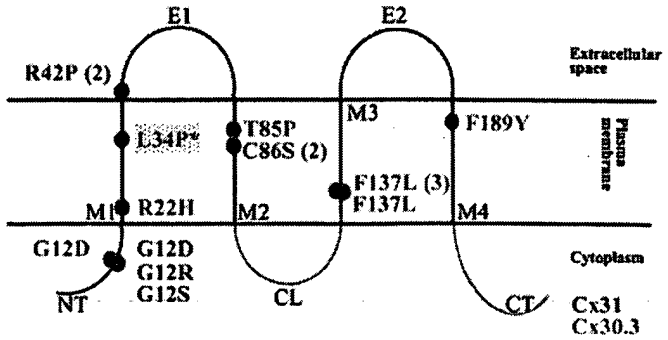

Figure 6. Schematic representation of connexin Cx31 and Cx30.3 polypeptides depicting the predicted structural motifs and location of pathogenic mutations in the corresponding genes *GJB3* (*blue*) and *GJB4* (*red*) in EKV. NT, cytoplasmic amino-terminus; M1–M4, transmembrane domains; E1, E2, extracellular domains; CL, cytoplasmic loop; CT, cytoplasmic carboxy-terminus. Domains depicted as black lines are evolutionary conserved, whereas gray lines indicate variable protein domains. Heterozygous dominant mutations in *GJB4* (Cx30.3) are shown as filled red circles, whereas mutations in *GJB3* (Cx31) are depicted as blue filled circles (Richard, 2001; Richard *et al*, 1998a; 2000; Wilgoss *et al*, 1999; Macari *et al*, 2000). The asterisk and shaded background annotate a recessive mutation (Gottfried *et al*, 2002).

reported in EKV (five out of 13) involve one of two conserved phenylalanines in M3 or in M4, suggesting a common pathomechanism (Wilgoss *et al*, 1999; Macari *et al*, 2000; Richard *et al*, 2000). Comparisons of channel kinetics of connexins with a different number of phenylalanines in M3 suggested that a loss of a phenylalanine residue might result in faster closure of gap junction channels, which could possibly alter Cx31/Cx30.3 mediated intercellular communication (Macari *et al*, 2000). It remains difficult to predict how the other mutations interfere with connexin structure and proper topogenic assembly of connexon hemichannels as their three-dimensional atomic structure has not been completely solved. In general, we speculate that pathogenic defects of Cx31 and Cx30.3 exert a dominant negative effect on the wild-type alleles, as has been demonstrated *in vitro* for dominant mutations in Cx26 (Richard *et al*, 1998b; White *et al*, 1998; Rouan *et al*, 2001) and Cx50 (Pal *et al*, 1999), and potentially on the function of selected other connexin species (Rouan *et al*, 2001; M. Falk, personal communication). Alternatively, survival of cells expressing mutant connexins might be impaired (Di *et al*, 2002; Diestel *et al*, 2002). Such mechanisms could explain why mutations in Cx30.3 or Cx31 cannot be fully compensated by other epidermal connexins *in vivo* and therefore result in a disease phenotype.

Defying our efforts, a group of seven EKV families had no detectable pathogenic mutations in either *GJB3* or *GJB4*, which, together with a recently reported case (Ishida-Yamamoto *et al*, 2000), amounts to about one-third of all families tested. In our cohort, no specific clinical characteristics could be identified that would help to recognize these patients based on clinical grounds. These families could harbor small nucleotide changes in the first, noncoding exons or regulatory gene regions, although such mutations were rarely described in other connexin disorders (The Connexin-Deafness Homepage). Another possibility is the presence of large genomic deletions within the connexin gene cluster on 1p35.1, which is supported by the recent discovery of two, possibly identical, partial deletions of *GJB6* in autosomal recessive nonsyndromic hearing loss (DFNB1) (Lerer *et al*, 2001; del Castillo *et al*, 2002). These *GJB6* deletions, one spanning 342 kb, were found to be a frequent cause of hearing loss *in trans* with another recessive mutation in *GJB2* or *GJB6* (del Castillo *et al*, 2002). Finally, mutations in yet another disease gene might be implicated in the pathogenesis of EKV.

Though not unusual for connexin genes, we identified a relatively large number (five) of nucleotide variants in the coding sequence of *GJB4*. The high allelic frequency of deletion 154del4 in our control group (3.3%) corresponds well with data obtained in a cohort of 243 hearing-impaired individuals (4.1%) as well as 69 normal controls (4.5%) from Spain (Lopez-Bigas *et al*, 2002). One individual who was homozygous for this null allele had early onset hearing loss, but 154del4 did not cosegregate with hearing loss in several other families. None of the carriers of 154del4 in either population group had a cutaneous phenotype, suggesting that it is probably a sequence polymorphism and that the absence of Cx30.3 in the skin might be compensated by other connexins. Neither study provided evidence that the deletion variant occurs in a *GJB4* pseudogene, though this possibility has not been completely excluded. We also identified three heterozygous amino acid substitutions (R124Q, C169W, and E204A) in control individuals without skin findings, whereas Lopez-Bigas *et al* (2002) detected these variants exclusively in small families or sporadic cases with hearing loss but not in control individuals of normal hearing (**Table IV**). No homozygous carrier of either variant has been identified to date. Nonetheless, most observed sequence variants reside within highly conserved protein domains, and, in particular, replacement of Cys169 can be expected to be detrimental to the structural stability of the extracellular portion of Cx30.3 connexons and to their ability to form complete gap junction channels (Foote *et al*, 1998). Considering our findings and disputes over the biologic relevance of amino acid replacements in other connexin genes, such as M34T in *GJB2* and R32W in *GJB3* (Kelsell *et al*, 2001; Lopez-Bigas *et al*, 2001; Di *et al*, 2002; Rouan *et al*, in press), it will be necessary to carefully investigate by functional assays whether these variants represent nonconsequential polymorphisms or recessive mutations.

Correlating our molecular findings with clinical data disclosed interesting genotype–phenotype correlations. (i) Despite the genetic heterogeneity of EKV, the clinical expression of *GJB4* mutations was similar to those in *GJB3*. With one exception, we found that all patients showed either coexistence of localized-symmetric or generalized hyperkeratosis and transient, variable erythematous patches, or, if the latter component was missing, they had a prior history of transient erythema (**Table I**). (ii) Compared to the severe, generalized hyperkeratosis documented in patients heterozygous for mutations G12D and F137L in *GJB3* (Richard, 2001), the corresponding mutations in *GJB4* appeared to be associated with a milder, localized involvement (**Table I**). Another *GJB4* mutation (F189Y), however, did not follow this trend, illustrating the need for continued investigations in a larger cohort of EKV patients. (iii) Independent of the segregating mutation in *GJB4* (**Table I**) or *GJB3* (Richard, 2001), severity and dominating features of disease strikingly varied within families (i.e., EKV2 or EKV3) but also during an individual's course of disease. In young children, the erythematous component usually prevailed (**Figs 2*B*, *E*, 4*F*, *G***), whereas hyperkeratosis was the dominant or sole feature in adults (**Figs 1*B*, 2*C***). We also identified a person (EKV2-2) without obvious cutaneous findings although harboring mutation T85P, but characteristic disease-associated erythema could be induced by cold exposure. Collectively, these data illustrate the marked, in part age-dependent, clinical variability of EKV that makes genotype–phenotype correlations difficult, and suggest the influence of other, yet to be identified, genetic and environmental factors modifying the clinical phenotype in EKV. (iv) Taking into account the clinical variability of EKV, the only appreciable phenotypic difference between individuals harboring mutations in *GJB3 versus GJB4* was the occurrence of rapidly changing, targetoid, or garland-like erythema with circinate borders in patients with faulty Cx30.3. Such lesions resembling erythema gyratum repens were first described in a Kurdish EKV family segregating with the *GJB4* mutation F137L (Macari *et al*, 2000). We observed this feature in four children and one adolescent of two unrelated EKV families (EKV2 and EKV11) harboring mutations T85P and F137L, respectively (**Figs 2*B*, *E*, 4*F*, *G***), whereas it was absent in all reported families with *GJB3* mutations (Wilgoss *et al*, 1999; Richard, 2001). These data suggest that circinate or gyrate erythema may be a specific manifestation of *GJB4* mutations, although the molecular and biologic mechanisms leading to localized capillary dilatation in EKV are elusive.

In summary, we have elucidated the molecular basis of EKV in six unrelated families and identified five new pathogenic missense mutations and a series of common sequence variants in *GJB4*. Our results establish the genetic heterogeneity of EKV and demonstrate the highly variable intrafamilial expression of *GJB4* mutations, which may include short-lasting erythematous patches with circinate or gyrate borders.

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MUTATION IN BRIEF

A Common Frameshift Mutation and Other Variants in GJB4 (Connexin 30.3): Analysis of Hearing Impairment Families

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Mutations in *GJB1*, *GJB2*, *GJB3* and *GJB6* are involved in hearing impairment. *GJB2*, *GJB3* and *GJB6* are also mutated in patients with hyperproliferative skin disorders. The human *GJB4* gene has been deduced in silico and a mutation in a family with erythrokeratodermia variabilis has been reported. We describe here the analysis of the *GJB4* gene in hearing impairment patients and control subjects. We have identified a common (4%) frameshift mutation (154del4) in *GJB4* in both affected and hearing subjects, one patient being homozygous for the mutation. We have also detected five amino acid variants (R103C, R124Q, R160C, C169W and E204A) in individuals that have not skin disorders. While mutation 154del4 is not associated with hearing impairment the involvement of some of the amino acid variants detected here is uncertain. These *GJB4* variants should help to define the putative role of connexin 30.3 in both skin disorders and hearing impairment.

KEY WORDS: Gap Junction; SNP; connexin 30.3; GJB4; hearing impairment; deafness; erythrokeratodermia variabilis; EKV

INTRODUCTION

Connexins are the protein subunits of gap junctions, which are involved in the process of intercellular communication (Kumar and Gilula, 1996). They are a multigenic family that have highly conserved sequences between them, formed by four transmembrane domains linked by one cytoplasmic and two extracellular loops, with cytoplasmic C- and N-terminal ends. To date fourteen human connexin genes have been described, several of which have been associated with human disorders. Four genes causing deafness (*GJB1* (Cx32), *GJB2* (Cx26), *GJB3* (Cx31) and *GJB6* (Cx30)) encode connexin proteins (Rabionet et al., 2000) (http://www.iro.es/deafness) and distinct dominant mutations in three of these genes *(GJB3, GJB2* and *GJB6)* are involved in skin diseases (reviewed in Kelsell et al., 2001 and Richard et al., 2000). Recently a mutation (F137L) in *GJB4* (MIM# 605425) has been identified in affected members of a family with erythrokeratodermia variabilis (EKV; MIM#1 33200) (Macari et al., 2000) but the role of *GJB4* in skin disorders has to be confirmed with the identification of additional

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EKV families with *GJB4* mutations. Furthermore it is unknown whether, as in other epidermal disease-associated connexins, *GJB4* mutations also result in hearing impairment.

MATERIALS AND METHODS

For mutation analysis of coding region *GJB4* it was PCR amplified in two fragments. The sequences of the primers used were: 5'-ggtagcacccaggtatagacc-3' (AL121988: 71531-71551) and 5'-ggtgaagaccttcttctccgt-3' (AL121988: 70938-70958) for the first part of exon 2, 5'-ggtgaagaccttcttctccgt-3' (AL121988: 71098-71118) and 5'-gccctgccttttttcctggg-3' (AL121988: 70628-70649 to) for the second part of exon 2. PCR was performed in a 25 µl total volume, containing 100 ng of genomic DNA, 7.5 pmols of each primer, 250 µM of each dNTP, 1.5 mM $MgCl_2$. The conditions for the reactions were: 94°C for 5 min; 35 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 40 sec, and a final extension of 72°C for 5 min.

Variation from wild-type sequence was detected by SSCP/heteroduplex analysis, as described elsewhere (Sala and Espinosa-Parrilla, 1999). Direct automatic sequencing of variant fragments was performed with the same primers on an automatic genetic analyzer.

In the Italian families the 154del4 deletion was analyzed by by electrophoresis with Genescan and Genotyper using the following primers: 5'-ggtagcacccaggtatagacc-3' (AL121988: 71531-71551) and 3'-atagcagacgttggggca-6' (AL121988: 71305-71322).

RESULTS AND DISCUSSION

We have performed SSCP mutational analysis of the *GJB4* coding region in 243 Spanish subjects with hearing impairment. An abnormal SSCP band pattern was detected in 9 deaf patients and sequencing revealed a 4-bp deletion (154del4) (Fig. 1A). The analysis of this deletion in Italian deaf (312) and control (108) individuals revealed 13 and 4 subjects, respectively, with the 154del4 mutation. This deletion is predicted to result in a frameshift, leading to an altered amino-acid sequence from codon 52, followed by a stop at codon 106 (Fig. 1B). This deletion does not cosegregate with hearing loss in most of our families as shown for family S6 (Fig. 1C). In family S49 the only deaf member available for analysis has the 154del4 mutation in homozygosity. He has an early onset hearing loss without any other associated phenotype including skin alterations. 154del4 mutation analysis of control subjects revealed a similar frequency (8 in 177, 4.5%) to deaf subjects (Table 1).

We also identified by SSCP analysis five nucleotide changes leading to putative amino acid variants (R103C, R124Q, R160C, C169W and E204A) (Table 1) (Fig. 2). The sequence of exon 2 of *GJB4* in members of families S31 and SC11 showed a C to T transition at nucleotide 307, resulting in an arginine to cysteine change. The type of deafness in both families is different, while family S31 has a late onset hearing impairment; family SC11 has a congenital dominant hearing loss. In members of families S155 and SC1 a G to A transition at nucleotide position 371 results in a R124Q change, the mutation being present in both hearing and non-hearing members of family SC1. Families S31 and SC1 also have the mitochondrial mutation A1555G (Lopez-Bigas et al., 2000), which is more likely to be the cause of deafness in these families, since some members referred deafness after treatment with amino glycosides. Sequencing of exon 2 of *GJB4* in some members of family SC54 revealed a C to T transition at position 478, leading to a putative arginine to cysteine change at codon 160. Two members of family SC54 also have the 154del4 mutation, but these variants do not cosegregate with the hearing loss. The sequence of exon 2 of *GJB4* in the deaf patient of family S17 revealed a C to G change at position 507 that results in a cysteine to tryptophan change at position 169. Finally, in family S9 we have detected an A to C transition at nucleotide position 611, resulting in an E204A change. None of these families referred skin disorders.

One interesting observation of this study is the 4% carrier frequency of the frameshift 154del4 mutation in both deaf and control subjects, which is even higher than the 2.5% frequency of the deafness mutation 35delG in *GJB2,* accounting for 30-50% of cases of congenital deafness (Rabionet el al., 2000). Taking into account the high carrier frequency of 154del4, the probability of finding a 154del4 homozygote would be 1/2500. If the 154del4 mutation were responsible for the deafness phenotype and considering that 1/2000 of infants are born deaf due to genetic causes (Rabionet el al., 2000), 80% of deafness cases would be due to 154del4 homozygosity. Since we have only found one homozygous subject out of 555, we can conclude that the 154del4 mutation is not a cause of deafness. Furthermore the high frequency of the 154del4 mutation in the general population and the finding of an individual with the mutation in homozygosity and without any associated phenotype other than deafness indicate that the total absence of this gene is neither lethal nor causes an obvious phenotype.

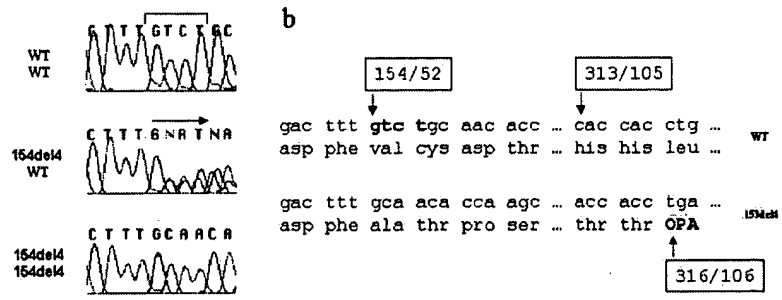

Fig. 1. 154del4 deletion in the *GJB4* gene in families with hearing impairment. **A** Partial sequence of *GJB4* in the region of mutation 154del4 showing the wild type (WT) sequence, the 154del4 in heteroygosity, and 154del4 in homozygosity. **B** Nucleotide and amino acid sequence of the normal and the 154del4 alleles. Deleted nucleotides are shown in bold. Boxes indicate nucleotide and codon positions. **C** Pedigree of two families with mutation 154del4.

Fig. 2. Pedrigrees of the families with amino acid variants and nucleotide sequence of a patient with the mutation and wild type sequence for each variant.

Table 1. Nucleotide and Amino Acid Changes in *GJB4* (Connexin 30.3) in Patients With Hearing Impairment and in Control Subjects

Nucleotide change	*Amino acid change*	*Domain*	*Deafness n (%)*	*Controls n (%)*
154del4	frameshift	EC1	23/555 (4.1)	8/177 (4.5)
307 C>T	Arg103Cys	IC2	2/243 (0.8)	0/69
371 G>A	Arg124Gln	IC2	2/243 (0.8)	0/69
478 C>T	Arg160Cys	EC2	1/243 (0.04)	0/69
507 C>G	Cys169Trp	EC2	1/243 (0.04)	0/69
611 A>C	Glu204Ala	C	1/243 (0.04)	0/69

We have also found five amino acid changes in different domains of the *GJB4* gene (Table 1) in seven families with deafness out of 243 families analyzed and we have not detected any of these variants in 69 control subjects (138 chromosomes). The five mutated residues are conserved in mouse and rat Cx30.3. C173 and E204 are also conserved in all the other human beta-connexins. In some of the families studied here the amino acid variants do not cosegregate with the deafness phenotype, but in others the family is not large enough to link or exclude these variants with deafness (Fig. 2). Although a mutation (F137L) in *GJB4* has been associated with EKV in one family (Macari et al., 2000), the individuals with amino acid changes in *GJB4* that we have reported here have no skin disorders. Further studies at the functional level and the analysis of other families will be necessary to elucidate the role of *GJB4* in skin disease and hearing impairment.

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